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(54) Title: METHODS FOR LABELING NUCLEOTIDES, LABELED NUCLEOTIDES AND USEFUL INTERMEDIATES

(57) Abstract

The present invention provides improved methods for labeling nucleotides. The method of the invention for labeling nucleotides comprises the steps of: reacting a reactive moiety of a linker, which linker is a platinum compound having a stabilizing bridge and two reactive moieties, with an electron donating moiety of a spacer, which spacer comprises a chain having at least four atoms and at least one heteroatom in the chain, which spacer further comprises said electron donating moiety at one end of the chain and a reactive moiety at the other end of the chain; reacting the reactive moiety of said spacer with a label; reacting the other reactive moiety of said linker with a nucleotide. A major advantage of the invention is that all nucleotides can be labeled by the method of the invention, whereas until now the attachment of a label was mostly restricted to one or certain nucleotides.

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Title: Methods for labeling nucleotides, labeled nucleotides and useful intermediates.

The invention relates to methods for labeling nucleotides using linkers (linking moieties between labels and bio-organic molecules, which linkers are based on platinum compounds).

Platinum (coordination) compounds have been considered interesting molecules for a very long time. For a review of these compounds and their uses we refer to Reedijk et al. (Structure and Bonding 67, p.53-89, 1987). Especially Cis-platinum has received a lot of attention as a possible anti-tumour drug. This anti-tumour reactivity of platinum compounds originates from their having at least two reactive groups (preferably cis-oriented towards each other), which make it possible to cross-link DNA molecules, thereby inhibiting the replication of these DNA molecules.

The British patent application 2 148 891 discloses cis-platinum complexes, which are six-coordinated. The platinum is attached to two halogens or hydroxy groups, two additional halogens and to an ethylene diamine derived group, such as 1,2-diamino-2-methylpropane or 1,2-diamino-2-methylbutane. The complexes are said to have an excellent anti-tumor effect.

In the European patent application four-coordinated complexes of platinum to 2,3-alkyl-1,4-butanediamine and two halogens are described for their anti-tumor effect.

Different four-coordinated platinum complexes are described in the European patent application 0 386 243. The complexes comprise a diamine bidentate ligand and two 2-arylalkanoic acid or 3-aryl-2-oxoalkanoic acid ligands. These complexes are stated to have a strong growth inhibiting action on certain leukemia cells and are used for their oncostatic activity.

US patent 4,207,416 discloses ethylenediamine-platinum(II) 2,4-dioxopyrimidine complexes as having a high anti-tumor activity and low mammalian toxicity.

A different use of platinum (coordination) compounds has been disclosed in PCT application (WO92/01699) wherein a platinum compound having only two reactive moieties (denominated as leaving groups therein) is reacted with a fluorescein to obtain a labeled platinum compound which can bind (non-covalently) to a nucleic acid, preferably at the N-7 position of a guanine residue.

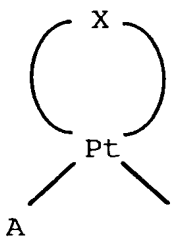
Several methods for labeling nucleotides have been described in the literature. For a long time, the standard method has been to use radioactive isotope labeling. However, there are a number of problems associated with the use of radioisotopes, such as potential health hazards, disposal problems and instability problems.

In order to overcome these problems, Dale et al., Biochemistry, **14**, (1975), 2447-2457, have proposed to use direct covalent mercuration as a labeling technique for nucleotides and polynucleotides. It was found, that cytosine and uracil may be mercurated at their C5-position under mild conditions. Further, Gebeyehu et al., Nucleic Acids Research, 15, (1987), 4513-4534, have reported that adenine and cytosine may be labeled with biotin derivatives through an aliphatic linker of from 3 to 17 atoms.

A major drawback of these known methods is that they are not suitable for labeling all different nucleotides. For instance, Dale et al. reported that their covalent mercuration method leads to negative results for adenine, thymine and guanine bases. In some cases, for example when only a few residues of a certain nucleotide are present in a certain nucleic acid or when the terminating nucleotide residue of a nucleic acid has to be labeled, it is desired to have at one's disposal a method for labeling any nucleotide residue.

The present invention provides such a method. The method for labeling nucleotides of the invention comprises the steps of:

- reacting a reactive moiety of a linker of the formula



(I)

wherein X represents any stabilizing bridge and wherein A and B represent the same or different reactive moieties, with an electron donating moiety of a spacer, which spacer comprises a chain having at least four atoms and at least one heteroatom in the chain, which spacer further comprises said electron donating moiety at one end of the chain and a reactive moiety at the other end of the chain;

- reacting the reactive moiety of said spacer with a label;
- reacting the other reactive moiety of said linker with a nucleotide.

According to the invention, the linker may first be attached to the nucleotide and then to the spacer, or vice versa and the spacer may first be coupled to the label and then to the linker or vice versa.

The reactive moiety of the spacer may be any reactive moiety that will enable the reaction between the spacer and the label in such a manner that a labeling moiety comrpising a label and a spacer is formed, which labeling moiety is sufficiently stable.

The main purpose for labeling nucleotides is that these labeled nucleotides can be incorporated in nucleic acid molecules. Modified nucleotides, especially those wherein a (bulky) label is attached to the nucleotide, are often built-in into nucleic acids with a much lower efficiency. The methods according to the invention result in labeled nucleotides which are built-in into nucleic acids with a higher efficiency than the labeled nucleotides available to date. This is probably for due to the selection of the spacers according to the invention in combination with the platinum-based linkers according to the invention.

The label to be used according to the invention is not critical. In principle all labels which can be attached to a nucleotide and are employed to date can be used. These labels may be radioactive labels, enzymes (which need reaction with a substrate to be detected), specific binding pairs components such as avidin, streptavidin or biotin, biocytin, iminobiotin, colloidal dye substances, fluorochromes (rhodamin, etc.), reducing substances (eosin, erythrosin, etc.), (coloured) latex sols, digoxigenin, metals (ruthenium), metal sols or other particulate sols (selenium, carbon and the like), dansyl lysin, Infra Red Dyes, coumarines (amino methyl coumarine), antibodies, protein A, protein G, etc. The invention has most benefits with bulkier labels such as biotin, avidin, streptavidin, digoxygenin or a functional equivalent thereof.

The invention is not limited to nucleotides or nucleosides as such; derivatives and functional equivalents are also included. The usual nucleotides adenine, thymidine, cytosine, guanine and uridine are preferred. Especially the purines are preferred which have a very good incorporation rate.

For coupling of the spacer to the platinum linker an electron donating moiety is required. In a preferred method the electron donating moiety is an amine or a thiolate anion, which have both proven to be very succesful. It was found that aromatic amines, such as imidazoles or purines, are capable of forming very strong bonds to platinum and thus are very suitable for use as the electron donating moiety.

The spacer is an important aspect of the present invention; it provides the easiest coupling between label and linker. For avoiding steric hindrance in incorporation of the nucleotide into the nucleic acid it should at least be four atoms long, preferably it is at least four carbon atoms long and has at least one heteroatom in that carbon chain. A heteroatom confers a certain amount of rigidity on the spacer. This rigidity provides an additional assurance that steric factors will not obstruct a convenient linking of a nucleotide and a label. It is preferred that at least one heteroatom is

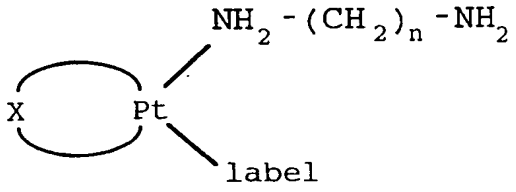
an oxygen atom, which positively effects the hydrophilicity of the spacer.

Preferably, the spacer comprises no more than 20 carbon atoms in the chain, which is preferably an essentially non-branched chain, thus causing no steric hindrance. The reason for this will be clear.

A highly preferred spacer is 1,8-diamino-3,6-dioxaoctane, herein referred to as Dadoo. Dadoo is a very flexible compound with a distal primary amine group and a size that makes it very suitable for use as spacer according to the invention.

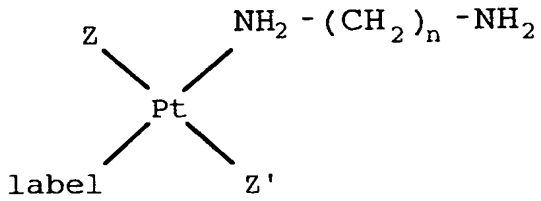
Another highly preferred spacer of the invention is an oligolysine or a polylysine. Due to their structure and conformation, these molecules create the most convenient environment for an optimal interaction among the actual label, the nucelotide and the platinum. An additional advantage of the use of lysine chains as the spacer is, that by altering the number of lysine units in the chain, the optimal conditions for specific labels and nucleotides or nucleic acids can be attained. Given a certain application, the skilled person will easily determine how many lysine units are required for optimum results.

An especially interesting labeling moiety comprising a label and a spacer, has the formula



(II)

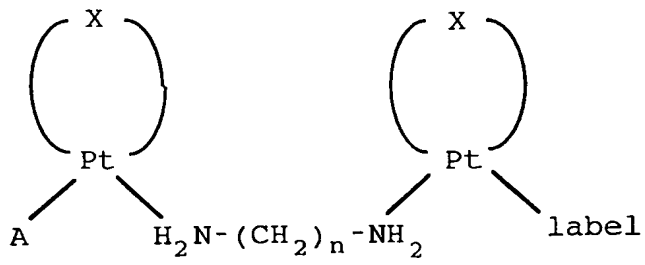
or the formula



(III)

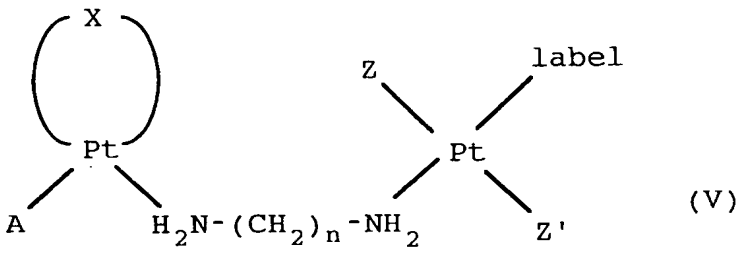
wherein X represents any stabilizing bridge, Z and Z' represent a non-leaving ligand and n is an integer of from 2 to 10.

Accordingly, the linker-spacer-label-system, or labeling substance, with the labeling moiety of formula (II) or formula (III) has the formula



(IV)

or the formula



(V)

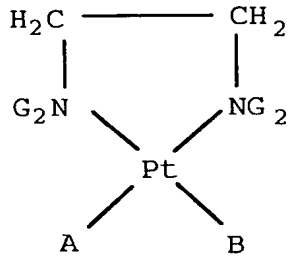
wherein A, X, Z, Z' and n have the above meanings.

The non-leaving ligands Z and/or Z' are preferably an NH_3, NH_2R, NHR_2 or NR_3 group, wherein R represents an alkyl group having from 1 to 6 carbon atoms, because these ligands have an even smaller leaving-group character than other non-leaving ligands.

The interesting feature of using the labeling moieties having formulas (II) and (III) is that both the nucleotide and the actual label have the benefit of being bonded directly to a platinum atom, while at the same time these moieties are sufficiently far apart to avoid steric hindrance.

The linkers according to the invention preferably are platinum compounds wherein X represents an aliphatic diamine. In a preferred embodiment of the invention, one or both of the nitrogen atoms of the aliphatic diamine are shielded. A suitable manner of shielding these nitrogen atoms consists of substitution with one or two alkyl groups of from 1 to 6 carbon atoms, preferably methyl groups. This is advantageous in that hydrogen bonding between the triphosphate group of a nucleotide and the stabilizing bridge is prevented. Preferably, a diamine having 2-6 carbon atoms is used, most preferably an ethylene diamine group, which is well-known for

its stabilizing effect on this class of platinum compounds. In this case, the linker has the formula



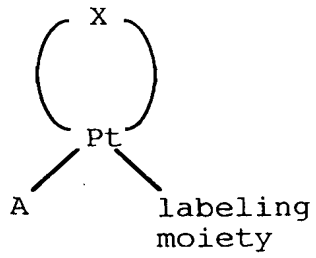
(VI)

wherein G represents hydrogen or an alkyl group of from 1 to 6 carbon atoms and A and B represent the same or different reactive moieties.

The coupling or reactive moieties A and B are preferably the same and selected from the group consisting of NO_3^-, SO_3^-, Cl^-, I^-, or other halogens.

The invention of course also encompasses a labeled nucleotide obtainable by a method as disclosed above.

In addition, the invention encompasses a labeling substance for labeling nucleotides by a method as disclosed above. The labeling substance of the invention has the formula



(VII)

wherein X and A have the above meanings and the labeling moiety comprises a label and a spacer as described above. Of course the labeling substances of the invention can also be used for labeling purposes other than labeling nucleotides. It was found that numerous (bio-) organic compounds, i.e. nearly every bio-organic molecule which contains an accessible

sulphur or nitrogen atom, for example proteins, can be labeled with the platinum compounds of the invention.

A great advantage of the invention arises from the use of the platinum compounds having formula (I) as linkers in the methods of preparing labeled nucleotides according to the invention. These linkers can be prepared by very convenient and reliable methods.

In WO92/01699 the starting compounds disclosed for preparing labeled platinum compounds are platinum(II)(ethylenediamine)dichloride and platinum(II)(ethylenediamine)(Me_2SO)Cl. The first one can be obtained commercially, the second one (the preferred one) must be synthesized. In the dichloride compound, the Cl-ions are less readily substituted by a label or a nucleotide, respectively. In the latter case, the total nucleotide labeling time will be appreciably longer, up to several hours, instead of several minutes.

The methods for preparing the linkers that are used in the method of labeling nucleotides according to the invention are based on the selection of suitable starting compounds of the formula PtE_4 wherein E is an electronegative group, preferably a halogen or NO_3^- or SO_3^-. The reaction, which is described in the examples, of these starting compounds with e.g. ethylenediamine is very simple and efficient. Moreover, this reaction leads to very suitable symmetric intermediate compounds for producing labeled nucleotides. A major advantage of using these compounds is that when a stabilizing bridge for the resulting platinum compound has to be attached that no blocking reagents have to be employed. Another advantage is that the resulting intermediate compounds can again be labeled without the use of blocking agents. Therefore steps removing blocking agents can be eliminated completely. Furthermore the yields of these reactions are very high. Yet another advantage of the use of these symmetrical starting compounds is that no mixtures of different resulting compounds can be formed, which may interfere with the following reaction and reduce yield or require extra separation steps.

A very suitable intermediate compound according to the invention is platinum(II)(ethylenediamine)$(NO_3)_2$. This substance can very easily be provided with a suitable spacer and a labeling group, resulting in labeling substances which can, through substitution of the remaining NO_3-group be linked to a nucleotide quite easily. Furthermore the methods for producing these compounds and the resulting compounds do not involve highly toxic substances such as DMSO.

The intermediate compounds can be labeled with any suitable label (also known as marker) through a spacer as disclosed hereinabove.

Furthermore, the known advantages (from WO92/01699 for instance) are of course also obtained with the present methods and compounds. Another advantage of the platinum compounds is that they can be detected more or less directly by using the platinum as a nucleus for depositing silver or other metal crystals.

By binding the labeling substance to a nucleotide residue, DNA or RNA molecules, be it single stranded or otherwise, can be easily detected, but it also allows for the production of probes for hybridization techniques wherein unlabeled DNA/RNA molecules hybridize to the labeled probe. The platinum linker labeled nucleotides do hardly interfere with the hybridization, if at all. Also, this technique obviates the use of modified nucleotides in preparing probes.

Nucleotides modified in accordance with the practices of this invention and oligo- and polynucleotides into which the modified nucleotides have been incorporated or oligo- and polynucleotides that have been directly modified using these novel platinum compounds may be used as probes in biomedical research, clinical diagnostics and recombinant DNA technology.

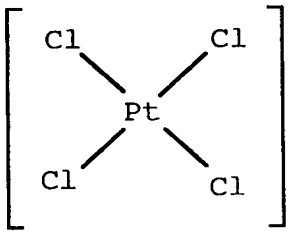
Other advantages and embodiments of the invention will become clear from the following experimental part and the examples.

EXPERIMENTAL

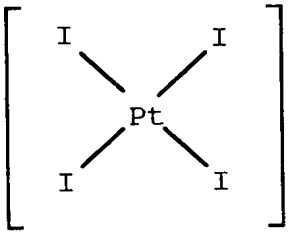
SYNTHESIS OF INTERMEDIATE PLATINUM COMPOUNDS

These compounds, i.e. the linkers having formula (I), may be prepared by a process which involves:

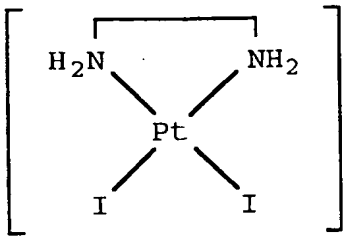
(a) reacting a compound having the structure:



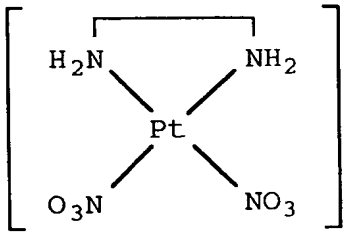
with KI in a suitable solvent under suitable conditions so as to form a iodated platinum compound having the structure:



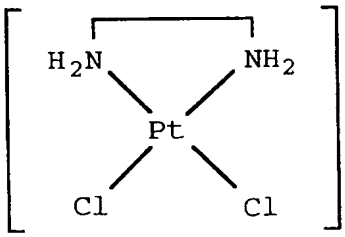
(b) reacting said iodated platinum compound with ethylenediamine in a suitable solvent so as to form a diethyleneamine iodated platinum compound and represented by the formula $Pt(en)I_2$ and having the structure:



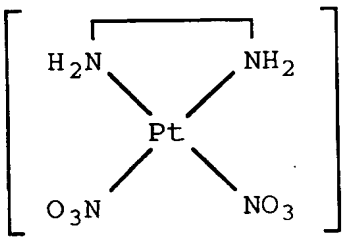
(c) reacting said compound with $AgNO_3$, the reaction being carried out in a suitable solvent, under suitable conditions so as to form a compound having the structure:



(d) reacting said compound with KCl in a suitable solvent under suitable conditions so as to form a compound having the structure:



(e) reacting said compound with $AgNO_3$ in a suitable solvent, under suitable conditions so as to form a compound having the structure:



(f) recovering said compound as modified platinum starting compound for the synthesis of hapten-bound Pt(en) compounds for use as DNA and/or RNA label.

Example 1

A. Preparation of Pt(en)-diamine starting material.

Preparation of Platinumethylenediamine$(NO_3)_2$: starting material.
$Pt(en)(NO_3)_2$

All reactions are performed in the dark.

Dissolve 1 gram potassium tetrachloroplatinate (II), K_2PtCl_4 (2.4 mmol, Sigma) in 50 ml millipore (filtered water) and stir at room temperature. Add 10 equivalents of potassium iodide, KI (24 mmol, 3.999 g, Sigma). The colour of the solution will immediately turn from orange into dark red (K_2PtI_4), stir for 5 minutes.

Add one equivalent ethylenediamine (2.4 mmol, 160.8743 µl, Merck 1l=0.9 kg) after diluting 161 µl ethylenediamine in 5 ml millipore very slowly to the platinum solution, mix this solution for 1 hour at room temperature.

A yellow/brown precipitate, $Pt(en)I_2$, will be formed and the liquid standing above should be clear.

Filter the solution through a 1.0 µm membrane filter (Schleicher&Schuell), wash the precipitate with millipore, ethanol and diether (in this order). Dry the $Pt(en)I_2$ for at least 4 hours in a vacuum dryoven at 37°C.

Weigh the dried $Pt(en)I_2$ (~1.07 g) and suspend it in 45 ml millipore/5 ml aceton, the solution will be cloudy. Add 1.95 equivalent of $AgNO_3$ (M = 169.9, Sigma). Stir the reaction overnight at room temperature.

Filter the solution through a 1.0 µm membrane filter, the precipitate is Silveriodide, AgI, the filtrate should be clear.

Add to 0.5 ml of the filtrate, $Pt(en)(NO_3)_2$, an excess of KCl or NaCl and make sure that no white precipitate is formed immediately after adding the excess of KCl or NaCl. If no white precipitate (only a yellow one) is formed than add an excess of KCl or NaCl to the entire filtrate. After the yellow precipitate is formed filter the solution and wash the precipitate ($Pt(en)Cl_2$) with millipore, ethanol and diether.

Dry the precipitate for at least 4 hours in a vacuum dryoven at 37°C.

Weigh the dry $Pt(en)Cl_2$ (M=326,1), and suspend it in 45 ml millipore/5 ml aceton and stir the cloudy suspension. Add 1.95 equivalent $AgNO_3$ and stir the solution overnight at room temperature. The colour of the solution will become white, due to the formation of AgCl.

Filter the solution in the dark and evaporate the filtrate to remove the aceton by rotation evaporation untill 25 ml of the filtrate is left. The filtrate is then freezedried. The product is checked by NMR or Infrared Absorption Spectroscopy.

B. Preparation of Pt(tmen)-diamine starting material.

Preparation of Platinum-N,N,N',N'-tetramethylethylenediamine$(NO_3)_2$: starting material. Pt(tmen)$(NO_3)_2$

All reactions are performed in the dark

Repeat the entire procedure of Example 1A, but use N,N,N',N'-tetramethylethylenediamine instead of ethylenediamine.

Example 2

A. Preparation of [Pt(en)(BioDadoo-NH_2)(NO_3)](NO_3)

Dissolve Pt(en)$(NO_3)_2$ (18.2 mg, 0.048 mmol) in 10 ml of Millipore water and heat until dissolving. Dissolve BioDadoo (20 mg, 0.053 mmol, purchased from Boehringer Mannheim) in 5 ml of Millipore water. Add the two solutions together and adjust the pH to 8 by 0.1 N NaOH, react for at least 3 hours at 50°C. Isolate the end product by freeze drying.

B. Preparation of [Pt(tmen)(BioDadoo-NH_2)(NO_3)](NO_3)

Dissolve Pt(tmen)(NO_3)$_2$ (35 mg, 0.08 mmol) in 12.5 ml of Millipore water and heat until dissolving. Dissolve BioDadoo (32 mg, 0.085 mmol) in 10 ml of Millipore water. Add the two solutions together and adjust the pH by 0.1 N NaOH, react for at least 4 hours at 50°C. Isolate the end product by freeze drying.

C. Preparation of [Pt(en)(DigDadoo-NH_2)(NO_3)](NO_3)

Dissolve Pt(en)(NO_3)$_2$ (5 mg, 0.013 mmol) in 5 ml of Millipore water and heat until dissolving. Dissolve DigDadoo (9 mg, 0.016 mmol, purchased from Boehringer Mannheim) in 5 ml of Millipore water. Add the two solutions together and react for at least 4 hours at 50°C. Isolate the endproduct by freeze drying.

Example 3

A. Preparation of a labeled dGTP

Dissolve [Pt(en)(BioDadoo-NH_2)(NO_3)](NO_3) (9 mg, 0.012 mmol) in 2 ml of Millipore water. Add 2'-deoxyguanosine-5'-triphosphate (2.3 mg, 0.004 mmol) and adjust the pH to 6. Incubate for 24 hours at 50°C, freeze-dry and redissolve in Millipore water (1 ml) and filter through a membrane filter. Apply the mixture to a FPLC with MonoQ and purify with a linear gradient from 100% Millipore water to 100% 1M NH_4HCO_3, collect and pool appropriate fraction and isolate by freeze drying. Dissolve the product in a 100 mM solution of triethylamine ammonium acetate (TEAA) (1 ml) and apply to a Reversed Phase HPLC (C18) with a linear gradient from 100% 100 mM TEAA to 50% 100 mM TEAA / 50% 100 mM TEAA/acetonitrile (1/1 v/v), collect and pool the appropriate fraction and remove solvents by repeated evaporation in vacuo. Pass the product overa cation exchanger

(Dowex) in the lithium form, isolate the product by freeze drying.

B. Preparation of a labeled 5-AA-dUTP

Dissolve [Pt(en)(BioDadoo-NH_2)(NO_3)](NO_3) (6 mg, 0.008 mmol) in 2 ml of Millipore water. Add 2'-deoxyuridine-5-aminoallyl-5'-triphosphate (2 mg, 0.004 mmol) and adjust the pH to 8. Incubate for 24 hours at 50°C, freeze-dry and redissolve in Millipore water (1 ml) and filter through a membrane filter. Apply the mixture to a FPLC with MonoQ and purify with a linear gradient from 100% Millipore water to 100% 1M NH_4HCO_3, collect and pool appropriate fraction and isolate by freeze drying. Dissolve the product in a 100 mM solution of triethylamine ammonium acetate (TEAA) (1 ml) and apply to a Reversed Phase HPLC (C18) with a linear gradient from 100% 100 mM TEAA to 50% 100 mM TEAA / 50% 100 mM TEAA/acetonitrile (1/1 v/v), collect and pool the appropriate fraction and remove solvents by repeated evaporation in vacuo. Pass the product overa cation exchanger (Dowex) in the lithium form, isolate the product by freeze drying.

Example 4

Reaction for coupling Pt(en)-compounds to DNA

Typical reaction for labeling DNA molecules with a Pt-compound according to the invention.

5 µg of double stranded DNA is sonicated or DNase treated to yield fragments of 300-500 bp. 6 µg of Pt(en)-compound is added and the volume is adjusted to 50 µl with demineralised water. The reaction mixture is incubated at 65°C for 1 hour. Non-bound Pt(en)-compound is blocked by adding 100 µl of a NADDTC solution. The Pt(en)-compound labeled DNA is purified on a sphadex G-50 column. Readily labeled and purified DNA is stored at -20°C or used directly in a DNA probe based assay.

Pt(en)-compound labeled DNA probes can be stored at least 2 years at -20°C without loss of activity and/or specificity. All applications mentioned are carried out with probes labeled according to this protocol.

Example 5

Biotin labeling of DNA probes with [Pt(en)(BioDadoo-NH_2)(NO_3)](NO_3) (BioDadoo-ULS).

Introduction

The labeling method has been used to label DNA probes with Biotin for In Situ Hybridization (ISH). In this example labeling procedures including the protocols and data for quality control procedures are presented. For Biotin labeling a plasmid cloned total DNA of Human Papilloma Virus type 6 (HPV-6, 40% GC basepairs) was used.

Experimental procedures

Plasmid DNA preparation

Total DNA of Human Papilloma Virus type 6 was cloned into vector pSp-64. Plasmid DNA was transferred into E.coli (C-600) and plated onto ampicillin containing LB plates Single colonies were grown overnight in large culture.
Plasmid DNA was isolated according to the method of Birnboim and Doly[1], purified by Sepharose C1-2B columnchromatography (Pharmacia) and checked for inserts by restriction-enzyme-analyses. Plasmid DNA concentration was determined by A260/280 absorbtion. After ethanol precipitation the DNA was reconstituted in 10mM TRIS/HCl pH 7.2, 0.3mM EDTA to a final concentration of 1 µg/µl (batch# 150894). Subsequently this DNA was sonicated (Soniprep 150., MSE) for 3 times 10 minutes (amplitude 5 microns) on ice.

The size of the resulting DNA fragments was determined by 2% agarose gel electrophoresis and found to be in between 200-400 basepairs (batch# 051094).

Plasmid DNA labeling and purification

Plasmid HPV-6 DNA was labeled with BioDadoo-ULS by mixing the following reagents:

plasmid HPV-6 DNA (batch# 051094)	5 µl (1 µg/µl)
BioDadoo-ULS labeling reagent (batch# BX940830)	8 µl (1 µg/µl)
Demineralised water (<0.2/ µS/cm)	37 µl

The 50 µl reaction mixture was incubated for 15 minutes at 85°C.

Excess of labeling reagent was captured by adding 50 µl sodium diethyldithiocarbamate (2% solution in demineralised water) and incubating for 30 minutes at room temperature.

Unbound BioDadoo-ULS was removed, using a S300 HR microspin column (Pharmacia), by size exclusion chromatography.

Eluent volume was adapted to 500 µl giving a 10 ng/µl biotin HPV-6 probe concentration (batch# 061094).

Quality control for detection limits

The detection limit of the biotin probe of the invention was determined by direct spot blot and reversed filterhybridization according to the following protocols:

Direct spot blot

HPV-6 probe (batch# 061094) labeled with biotin according to the invention was 10-fold serially diluted into spot buffer comprising 900mM sodium chloride, 90mM sodium citrate and 200 µg/ml single stranded salmon sperm DNA giving a dilution series varying from 1000-0.1 pg biotin probe per µl.

1 µl spots were applied onto nitrocellulose membrane and incubated for 2 hours at 80°C to bind the DNA. The biotin probe was visualized using a streptavidin-alkaline phosphatase conjugate (Sigma) combined with a NBT/BCIP precipitating

substrate solution (Sigma) according to the following protocol:

- Membranes were soaked in TBS solution containing 0.5% tween20 (TBST) for 5 minutes.
- Membranes were incubated with Strep-AP (3 DEA U/ml) in TBST for 15 minutes at 37°C.
- NC-membranes were washed 3 times 5 minutes in TBS solution followed by a 5 minute wash step in demineralised water.
- Membranes were incubated with NBT/BCIP substrate solution for 15 minutes at 37°C, subsequently washed in demineralised water and air dried.

Results

By using this method the detection limit of the biotin DNA probe according to the invention was found to be less than 1 pg.

Reversed filterhybridization

HPV-6 DNA (batch# 051094) was1 in 10 diluted in 0.1N NaOH, incubated at 100°C for 5 minutes and directly placed on ice for 5 minutes to make DNA single stranded.

A 10-fold serial dilution was made in cold 0.1N NaOH to give a series varying from 10,000-1pg DNA per µl. 1 µl spots were applied onto Nylon membrane (Boehringer Mannheim) and air dried.

HPV-6 DNA probe that was labeled with biotin according to the invention was diluted in 5xSSPE 0.5% SDS solution to yield a concentration of 200 ng/ml.

This sloution was incubated for 5 minutes at 100°C and placed directly on ice for 5 minutes.

Nylon membranes containing target DNA were soaked in 2x SSC for 5 minutes and subsequently incubated with the single stranded probe solution for 2 hours at 37°C.

Excess of the biotin probe was removed by three changes in 2x SSPE 0.1% SDS for 10 minutes at 37°C followed by a 5 minutes TBST incubation.

The biotin probe of the invention was visualized by performing the same protocol as described in the direct spot blot method.

Results

By using this procedure the detection limit of the biotin probe according to the invention was found to be less than 10 pg.

Performance in In Situ Hybridization

The test material consisted of 6 µm paraffin sections of a HPV-6 posotive cervical condyloma mounted on organosilane coated glass slides.

The following protocol was applied (unless otherwise stated steps are at room temperature):

1 Paraffin sections were dewaxed in 3 changes of xylene and hydrated in graded ethanol.

2 Sections were rinsed in TBST for 5 minutes.

3 Sections were digested in 0.25% pepsin in 0.1N HCl for 30 minutes at 37°C, dehydrated in graded ethanol and air dried.

4 10 µl of probe solution was applied to a section and covered with a coverslip.

Probe solution consisted of biotin HPV-6 probe DNA labeled according to the invention (batch# 061094) in a concentration of 2 ng/µl dissolved in hybridization mixture comprising 0.6M NaCl, 0.06M sodium citrate, 35% formamid, 10% dextransulphate, 2,5x Denhardts and 10 µg/ml single stranded salmon sperm DNA.

5 Slides were placed on a hot plate set at 95°C for 5 minutes to denature probe and target DNA simultaneously.

6 Hybridization was performed by placing the slides in a humidified chamber at 37°C for 2 hours.

7 Coverslips were removed and slides were washed in 3 changes of 15mM NaCl, 1.5mM sodium citrate and 5% formamid for 10 minutes at 37°C.

8 Slides were rinsed in TBST.

9 Sections were incubated with Streptavidin AP copnjugate (3DEA U/ml in TBST) for 15 minutes at 37°C.

10 Slides were washed in TBST (3x) and demineralised water (1x) for 5 minutes.

11 Sections were incubated with NBT/BCIP substrated solution for 15 minutes at 37°C.

12 Slides were washed in demineralised water (3x) for 1 minute and sections were mounted in glycerol/gelatin.

Results

By using the sections showed blue/purple precipitates at the sites of HPV-6 infected cells and minor background in the remaining tissue.

Conclusions

The results demonstrate that DNA labeled according to the invention has good detection limits. The present method is very well suited for research, routine and for industrial production of labeled nucleic acids, as the method is fast and easy to perform, very sensitive, and does not include any enzymaic step, which makes it highly reproducible and fitted for an overall low cost production. The method of the invention offers a useful alternative equaling conventional non-isotopic labeling methods.

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Applications

1. **The use of Pt-DNA linkers of the invention in the so called LIDIA technique: Linked DNA immuno Assay.**

The LIDIA technique enables the quantitative analysis of small amounts of DNA (or RNA) e.g. after a PCR amplification of the starting material. The technique is sensitive and

specific, due to the use of specific DNA(RNA) probes in accordance with the invention and easy to perform, because of the quick DNA(RNA) Pt-labeling steps of the invention.

Description of the technique:

The technique uses fast Pt labeling compounds of the invention to label DNA(RNA) probes

This technique is possible with 3 different approaches.

1. Linking DNA probes molecules to a surface by using a Pt compound in accordance with the invention which cross-link DNA molecules irreversibly to plastic, nylon or nitrocellulose. Detection of DNA targets can then be accomplished by using classically labeled DNA/RNA probes.(nick translation or chemical modification, random priming)
2. Linking a detectable group to the DNA, to render a DNA molecule into a so-called DNA probe. Binding of DNA compounds to a surface can then be accomplished by using classic techniques known to science (covalent linking to specially treated microtiter plates, baking of DNA molecules onto nitrocellulose or binding of DNA molecules to nylon membranes.
3. A combination of techniques 1 and 2

Approach 1

An immobilized DNA probe can be used to catch specific target molecules in a sample by using a hybridization technique. Detection of formed hybrids can be done by using different techniques, e.g. a second labeled DNA probe can be used to hybridize with a different site on the target DNA molecule to form a sandwich hybrid. The label can then be detected by using state of the art immunological detection and colouring techniques.

Approach 2

A volume containing (amplified) detectable DNA(RNA) is directly labeled according to the protocol in accordance with the invention.

Excess label is quenched by adding NaDDTC or Thioureum. This approach distinguishes itself from other techniques by the fact that the target molecule is labeled in contrast to other assay were labeled DNA(RNA) probes are used to detect the target. The quick binding capacity of the Pt-label compound of the invention enables a DNA binding step as a routine step in a diagnostic test procedure (normal binding times are 60 minutes at 65°C).

A second step is performed in a microtiter plate precoated with a target specific probe. Incubation is allowed to the formation of stable "Labelled target" and probe hybrids. The direct labeling of target molecules enables the omission of laborious double hybridization techniques where one probe is used to catch the target and another labeled probe is used to detect the immobilized target.

In this method the probes are covalently linked to the microtiter plate to the surface of the wells. The second incubation step has the character of a liquid hybridisation and therefore can be performed very rapidly. This is one of the main innovative features of this approach to quantitive DNA hybridisation techniques.

Approach 3

Both for the immobilization of DNA probes or DNA targets and for the labeling of DNA probes and targets the newly developed Pt system can be used. These two DNA linking techniques can be combined into one assay where both the "catcher" and the "detector" are linked to a second substance (either a detectable group like biotin, digoxigenin or a carrier surface like a plastic stick, microtiter plate or a membrane).

Examples of the technique: the detection of STD related microorganisms in human diagnostics (Chlamydia, Syfilis, AIDS, Herpes, Gonorrhoea, Hep. B,)

2. The use of Pt-DNA labels of the invention in combination with test strip procedures and formats. The "DNA Dipstick".

The DNA dipstick technique enables the qualitative and semi-quantitative analysis of small amounts of DNA(or RNA) e.g. after a PCR amplification or freely present in samples of body fluids (blood, urine, sweat etc.)

The technique is sensitive and specific, due to the use of specific DNA(RNA) probes and easy to perform because of the quick DNA(RNA) Pt-labeling steps according to the invention.

The universal labeling characteristics of the newly developed Pt label can be used in 3 ways to achieve a bound DNA(RNA) molecule.

1. It can be used to attach a detectable marker group to a polynucleotide sequence.
2. It can be used to attach polynucleotide sequences irreversibly to a solid phase (plastic, membranes, latex beads, hydrosols or microtiter plate wells).

3. A combination of 1 and 2

ad 1:

In this example there is a twofold approach to the detection of biolytes biological analytes in test samples. Firstly a DNA probe can be labeled with the newly developed Pt labeling compound. This labeled probe can then be used to detect preformed hybrids on a membrane formed between the target DNA sequence and a primary probe. It is essential in this method that the primary probe recognizes a different sequence on the target than the secondary Pt labeled probe. In practice, this can be achieved for instance with RNA hybridization were a POLY A probe is used as a primary probe

to immobilize all RNA (recognizable by its polyT tails) to a membrane.

The second approach differs slightly in that in this case the target can be labeled in the test sample fluid, because of the fast and very specific Pt labeling characteristics. A procedure like this would comprise a catch of the labeled target with an immobilized specific unlabeled DNA probe on a suitable membrane. Hence a dipstick version for DNA/RNA applications.

ad 2:

To immobilize DNA probes or target DNA, a non-labeled Pt compound (that is a Pt compound with 2 free binding sites) can be used to act as a bridge between DNA and the surface of carriers (plastic, membrane, microtiter plates etc.)

It greatly enhances the usability of DNA sequences as catcher molecules in diagnostic assays, since there are little substances known to science that bind readily DNA in a spontaneous way. Introducing this Pt bridge molecule a wide field of new applications for the DNA technology has come within reach.

ad 3: a combination of example 1 and 2

General: the use of the Pt compound of the invention in latex or hydrosol assays is particularly interesting. The compound enables the coupling of DNA molecules to small particles. The DNA molecules can be hybridized to target material. A positive reaction is visualized by an agglutination of the particles, due to crosslinking of the DNA hybrid particle compounds.

A test like this can be made quantative, the rate of agglutination can be tuned and measured at a specific wavelength. Especially gold particles have the intrinsic characteristic that a shift in optimal wavelength occurs after agglutination.

3. Detection of Platinated DNA probes of the invention with the the silver-enhancement technique.

Platinated DNA/RNA probe can be employed in hybridisation methods to detect DNA/RNA sequences in sample material. The introduction of a platinum compound at the site of the target enables the deposition of Ag molecules in a chemical reaction especially designed to reduce ionic silver to metallic silver. At the site of a Pt nucleus a decomposition of metallic silver(black) occurs due to the catalytic effect of the Pt nucleus.

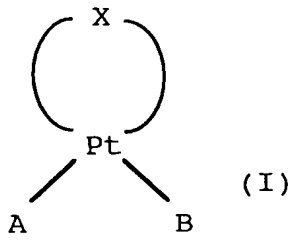
Ionic silver is reduced by a reducing agent (e.g. Na-borohydrid, Hydrochinon) in solution. In a constant ratio the amount of silver deposited on the Pt is proportional to the length of the enhancement incubation.
Visualisation of a non-visible Pt nucleus can be accomplished by the empirical observation of the appearace of a black spot in the test sample.

The black spots indicate the site of specific probes binding and thus the site of specific target location.
The technique enables a quick and easy diagnostic procedure for the detection of various microorganisms and gene translocations/abnormalities.

CLAIMS

1. A method for labeling nucleotides comprising the steps of:

- reacting a reactive moiety of a linker of the formula



(I)

wherein X represents any stabilizing bridge and wherein A and B represent the same or different reactive moieties, with an electron donating moiety of a spacer, which spacer comprises a chain having at least four atoms and at least one heteroatom in the chain, which spacer further comprises said electron donating moiety at one end of the chain and a reactive moiety at the other end of the chain;

- reacting the reactive moiety of said spacer with a label;
- reacting the other reactive moiety of said linker with a nucleotide.

2. A method according to claim 1 wherein the label is biotin, avidin, streptavidin, digoxygenin or a functional equivalent thereof.

3. A method according to claim 1 or 2 wherein the nucleotide is adenine, thymidine, cytosine, guanine or uridine or a derivative thereof.

4. A method according to any of the preceding claims wherein the electron donating moiety is an amine or a thiolate anion.

5. A method according to claim 4 wherein the amine is an aromatic amine.

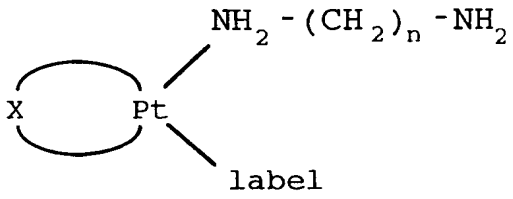
6. A method according to any of the preceding claims wherein at least one heteroatom is an oxygen atom.

7. A method according to any of the preceding claims wherein the spacer comprises no more than 20 carbon atoms in the chain and wherein the chain is essentially non-branched.

8. A method according to claim 7 wherein the spacer is 1,8-diamino-3,6-dioxaoctane.

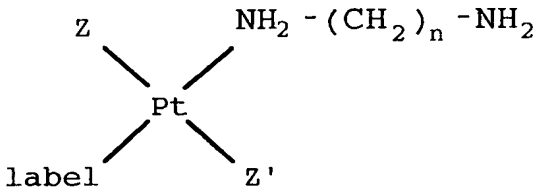
9. A method according to claim 7 wherein the spacer is an oligolysine or a polylysine.

10. A method according to claims 1-4 wherein the linker is reacted with a labeling moiety comprising a label and a spacer, which labeling moiety has the formula



(II)

or the formula



(III)

wherein X represents any stabilizing bridge, Z and Z' represent a non-leaving ligand and n is an integer of from 2 to 10.

11. A method according to claim 10, wherein Z and/or Z' represent an NH_3, NH_2R, NHR_2, or NR_3 group, wherein R represents an alkyl group having from 1 to 6 carbon atoms.

12. A method according to any of the preceding claims, wherein X represents an aliphatic diamine.

13. A method according to claim 12 wherein X represents an aliphatic diamine having 2-6 carbon atoms.

14. A method according to claim 13 wherein X is an ethylene diamine group.

15. A method according to claims 12-14 wherein one or both of the nitrogen atoms of the aliphatic diamine are shielded.

16. A method according to claim 15 wherein one or both of the nitrogen atoms of the aliphatic diamine are substituted with an alkyl group of from 1 to 6 carbon atoms.

17. A method according to claim 16 wherein one or both of the nitrogen atoms of the aliphatic diamine are substituted with one or two methyl groups.

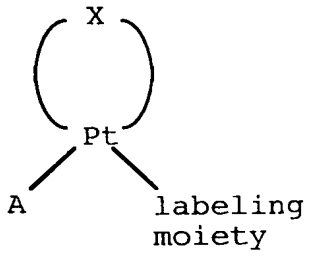
18. A method according to any of the preceding claims wherein A and/or B are selected from the group consisting of NO_3^-, SO_3^-, Cl^-, I^-, or other halogens.

19. A method according to any of the preceding claims wherein A and B are the same.

20. A labeled nucleotide obtainable by a method according to any of the preceding claims.

21. A labeled nucleotide according to claim 20 wherein the spacer is an oligolysine or a polylysine.

22. A labeling substance having the formula



(VII)

wherein X represents any stabilizing bridge, A represents a reactive moiety and the labeling moiety comprises a label and a spacer, which spacer comprises a chain having at least four atoms and at least one heteroatom in the chain, which spacer further comprises an electron donating moiety at the end of the spacer distal from the label.

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07F15/00 C07H21/00 C07H23/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1997	05/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Moreno, C

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According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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X	WO 92 01699 A (ACADEMISCH MEDISCH CENTRUM ;UNIV LEIDEN (NL)) 6 February 1992 cited in the application see the whole document ---	1,22
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A	GB 2 148 891 A (NIPPON KAYAKU KK) 5 June 1985 see the whole document ---	1,20,22
A	US 4 207 416 A (HOESCHELE JAMES D) 10 June 1980 see the whole document --- -/--	1,20,22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1997	05/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Moreno, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00559

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 282 672 A (NIPPON KAYAKU KK) 21 September 1988 see the whole document ---	1,20,22
A	EP 0 386 243 A (SAGAMI CHEM RES ;CHISSO CORP (JP)) 12 September 1990 see the whole document -----	1,20,22

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 97/00559

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201699 A	06-02-92	NL 9001639 A	17-02-92
		AT 145403 T	15-12-96
		AU 8286391 A	18-02-92
		DE 69123251 D	02-01-97
		DE 69123251 T	28-05-97
		EP 0539466 A	05-05-93
		ES 2097213 T	01-04-97
		US 5580990 A	03-12-96
WO 9635696 A	14-11-96	AU 5704096 A	29-11-96
GB 2148891 A	05-06-85	JP 1725766 C	19-01-93
		JP 4012277 B	04-03-92
		JP 60087295 A	16-05-85
		BE 900842 A	18-04-85
		DE 3438440 A	02-05-85
		DK 499584 A	20-04-85
		FR 2553777 A	26-04-85
		NL 8403087 A	17-05-85
		SE 8405188 A	20-04-85
		US 4607114 A	19-08-86
US 4207416 A	10-06-80	AU 8455375 A	10-03-77
		CA 1034120 A	04-07-78
		CH 617587 A	13-06-80
		DE 2539179 A	18-03-76
		FR 2283692 A	02-04-76
		GB 1526210 A	27-09-78
		JP 51054916 A	14-05-76
		SE 424870 B	16-08-82
		SE 7509769 A	08-03-76
		US 4080324 A	21-03-78
EP 0282672 A	21-09-88	JP 63203692 A	23-08-88
		CA 1292749 A	03-12-91
		US 4861905 A	29-08-89
		US 4987246 A	22-01-91
		US 5128493 A	07-07-92
EP 0386243 A	12-09-90	JP 2000294 A	05-01-90

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 97/00559

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0386243 A		JP 2033479 C	19-03-96
		JP 7062023 B	05-07-95
		JP 1249791 A	05-10-89
		WO 8904317 A	18-05-89

PATENT COOPERATION TREATY

09/402707

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
PCT 0630		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 97/00559	08/10/1997	08/10/1996
Applicant		
KREATECH BIOTECHNOLOGY B.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. ___–_____

   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

09/402707 19

PATENT COOPERATION TREATY

REC'D 29 DEC 1998
WIPO PC

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
PCT 0630		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL97/00559	08/10/1997	08/10/1996
International Patent Classification (IPC) or national classification and IPC		
C07F15/00		
Applicant		
KREATECH BIOTECHNOLOGY B.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/02/1998	29.12.98
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Friebel, F Telephone No. (+49-89) 2399-8552

Form PCT/IPEA/409 (Cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL97/00559

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-26 as originally filed

Claims, No.:

1-22 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-22
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-22
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-22
	No:	Claims

2. Citations and explanations

see separate sheet

point V:

The present application claims for a method for labeling nucleotides, the labeled nucleotides and intermediates (labeling substance of formula VII). The labeled nucleotide obtained according to the method claimed has the following basic structure:

nucleotide -Pt⊃ X - spacer - label

An important structural feature is the spacer which comprises a chain having at least four atoms and at least one heteroatom in the chain.

Closest prior art is the document **WO 92/01699 (D1)** which is already mentioned on page 2 of the application. This reference discloses a comparable type of Pt-containing labeling compounds which however differ in the spacer element. What is disclosed in this document is a quite specific thiourea spacer which neither anticipates nor makes obvious the spacer of the present application.

Furthermore, D1 does not disclose a combination of this labeling substance with a nucleotide. Novelty of the method claims as well as of the compound claims is therefore acknowledged. Since the D1 reference is silent on the labeling of nucleotides in a manner which allows the incorporation of the labeled nucleotides into a growing DNA or RNA chain, which in turn is a direct result of the spacer arm employed, inventive step is also acknowledged.

The subject-matter of Claims 1 to 22 meets the requirement of Art.33(2) and (3) PCT.

PATENT COOPERATION TREATY

09/402707

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SMULDERS, Th.A.H.J.
VEREENIGDE OCTROOIBUREAUX
Nieuwe Parklaan 97
2587 BN Den Haag
PAYS-BAS

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	29.12.98
Applicant's or agent's file reference PCT 0630	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL97/00559	08/10/1997	08/10/1996

Applicant
KREATECH BIOTECHNOLOGY B.V. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	DA ROCHA, O. Tel. (+49-89) 2399-8101

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
PCT 0630		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL97/00559	08/10/1997	08/10/1996
International Patent Classification (IPC) or national classification and IPC		
C07F15/00		
Applicant		
KREATECH BIOTECHNOLOGY B.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

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   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

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3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
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VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/02/1998	29.12.98
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Friebel, F Telephone No. (+49-89) 2399-8552

Form PCT/IPEA/409 (Cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL97/00559

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-26 as originally filed

Claims, No.:

1-22 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-22
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-22
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-22
	No:	Claims

2. Citations and explanations

see separate sheet

point V:

The present application claims for a method for labeling nucleotides, the labeled nucleotides and intermediates (labeling substance of formula VII). The labeled nucleotide obtained according to the method claimed has the following basic structure:

nucleotide -Pt⊃ X - spacer - label

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The subject-matter of Claims 1 to 22 meets the requirement of Art.33(2) and (3) PCT.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 16 April 1998 (16.04.98)	
International application No.: PCT/NL97/00559	Applicant's or agent's file reference: PCT 0630
International filing date: 08 October 1997 (08.10.97)	Priority date: 08 October 1996 (08.10.96)
Applicant: HOUTHOFF, Hendrik, Jan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

23 February 1998 (23.02.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

09/402707

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
PCT 0630		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 97/ 00559	08/10/1997	08/10/1996
Applicant		
KREATECH BIOTECHNOLOGY B.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

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   - [ ] furnished by the applicant separately from the international application,
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   - [ ] Transcribed by this Authority

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   - [ ] the text has been established by this Authority to read as follows:

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6. The figure of the **drawings** to be published with the abstract is:

   Figure No. –
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00559

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07F15/00 C07H21/00 C07H23/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 01699 A (ACADEMISCH MEDISCH CENTRUM ;UNIV LEIDEN (NL)) 6 February 1992 cited in the application see the whole document ---	1,22
P,X	WO 96 35696 A (KREATECH BIOTECH BV ;HOUTHOFF HENDRIK JAN (NL); REEDIJK JAN (NL);) 14 November 1996 see the whole document ---	1,20,22
A	GB 2 148 891 A (NIPPON KAYAKU KK) 5 June 1985 see the whole document ---	1,20,22
A	US 4 207 416 A (HOESCHELE JAMES D) 10 June 1980 see the whole document --- -/--	1,20,22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1997	05/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Moreno, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00559

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 282 672 A (NIPPON KAYAKU KK) 21 September 1988 see the whole document ---	1,20,22
A	EP 0 386 243 A (SAGAMI CHEM RES ;CHISSO CORP (JP)) 12 September 1990 see the whole document -----	1,20,22

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 97/00559

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201699 A	06-02-92	NL 9001639 A	17-02-92
		AT 145403 T	15-12-96
		AU 8286391 A	18-02-92
		DE 69123251 D	02-01-97
		DE 69123251 T	28-05-97
		EP 0539466 A	05-05-93
		ES 2097213 T	01-04-97
		US 5580990 A	03-12-96
WO 9635696 A	14-11-96	AU 5704096 A	29-11-96
GB 2148891 A	05-06-85	JP 1725766 C	19-01-93
		JP 4012277 B	04-03-92
		JP 60087295 A	16-05-85
		BE 900842 A	18-04-85
		DE 3438440 A	02-05-85
		DK 499584 A	20-04-85
		FR 2553777 A	26-04-85
		NL 8403087 A	17-05-85
		SE 8405188 A	20-04-85
		US 4607114 A	19-08-86
US 4207416 A	10-06-80	AU 8455375 A	10-03-77
		CA 1034120 A	04-07-78
		CH 617587 A	13-06-80
		DE 2539179 A	18-03-76
		FR 2283692 A	02-04-76
		GB 1526210 A	27-09-78
		JP 51054916 A	14-05-76
		SE 424870 B	16-08-82
		SE 7509769 A	08-03-76
		US 4080324 A	21-03-78
EP 0282672 A	21-09-88	JP 63203692 A	23-08-88
		CA 1292749 A	03-12-91
		US 4861905 A	29-08-89
		US 4987246 A	22-01-91
		US 5128493 A	07-07-92
EP 0386243 A	12-09-90	JP 2000294 A	05-01-90

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 97/00559

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0386243 A		JP 2033479 C	19-03-96
		JP 7062023 B	05-07-95
		JP 1249791 A	05-10-89
		WO 8904317 A	18-05-89